Foxp3+ T cells inhibit antitumor immune memory modulated by mTOR inhibition

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Running title: Immunity is enhanced by mTOR inhibition and Treg removal

Summary: Findings offer a preclinical proof-of-concept that T regulatory cell depletion will enhance antitumor immune memory stimulated by mTOR inhibition.

Key words: temsirolimus, mTOR, CD4 depletion, CD8 memory, regulatory T cells

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ABSTRACT

Inhibition of mTOR signaling enhances antitumor memory lymphocytes. However, pharmacologic mTOR inhibition also enhances regulatory T cell (Treg) activity. To counter this effect, Treg-control was added to mTOR inhibition in preclinical models. Tregs were controlled with CD4 depleting antibodies because CD4 depletion has high translational potential and already has a well-established safety profile in patients. The antitumor activity of the combination therapy was CD8 dependent and controlled growth of syngeneic tumors even when an adoptive immunotherapy was not used. Lymphocytes resulting from the combination therapy could be transferred into naïve mice to inhibit aggressive growth of lung metastases. The combination therapy enhanced CD8 memory formation as determined by memory markers and functional studies of immune recall. Removal of FoxP3 expressing T lymphocytes was the mechanism underlying immunological memory formation following CD4 depletion. This was confirmed using transgenic DEREG (*de*pletion of *reg*ulatory T cells) mice to specifically remove Foxp3+ T cells. It was further confirmed with reciprocal studies where stimulation of immunological memory due to CD4 depletion was completely neutralized by adoptively transferring tumor-specific Foxp3+ T cells. Also contributing to tumor control, Tregs that eventually recovered following CD4 depletion were less immunosuppressive. These results provide a rationale for further study of mTOR inhibition and CD4 depletion in patients.

INTRODUCTION

The immune system can provide protection against cancers. Effective immune stimulation produces long-lasting memory lymphocytes, capable of rapidly responding to repeat antigen challenge. The mTOR (mechanistic target of rapamycin) pathway is an important checkpoint that governs the formation of CD8 memory cells (1-3). In mouse models, decreased mTOR signaling promotes formation of CD8 memory cells that provide protection against bacteria (4), virus (1) or cancer (2, 3). This is surprising since rapamycin, which is the prototypic mTOR inhibitor, is considered an immunosuppressant and is widely used to prevent rejection of solid organ transplants. In murine models of renal cell carcinoma (RCC) and melanoma, pharmacologic mTOR inhibition had both immune stimulating and immune suppressing effects (2). However, the net effect resulted in decreased tumor growth. Therefore, mTOR inhibitors, which are already FDA-approved for clinical use, are a promising adjunct for use with cancer vaccines.

Strategies to limit immune suppression by mTOR inhibitors may make this class of drugs even more useful with cancer vaccines. Pharmacologic mTOR inhibition suppresses the immune system at least in part by enhancing CD4 regulatory T cell (Treg) activity (2, 5). Therefore we explored a combination therapy targeting the mTOR pathway and Tregs. The most reliable Treg marker is forkhead box transcription factor (FoxP3), which is specific for Tregs and is required for its function (6). Unfortunately, there is no clinical strategy for targeting FoxP3 expressing cells in patients. Therefore, an alternative strategy is to target CD25, which is expressed by the majority of Tregs. However, this strategy has limitations because some Tregs are CD25 negative. Furthermore, activated CD8 lymphocytes express CD25 and can be depleted by CD25 targeting strategies. In murine models, depleting CD25 expressing cells with αCD25 antibodies was effective in preventing tumor growth, but was not effective in treating established tumors (7-9) and has been shown to restrict adoptive immunotherapy (10, 11).

Another strategy uses an engineered protein that combines interleukin-2 and diphtheria toxin (denileukin diftitox, trade name Ontax) to target CD25 expressing cells. The approach has been tested in patients with renal cell carcinoma (RCC) or melanoma (12, 13), however clinical effectiveness was limited, possibly due to depletion of CD8 effector cells.

Using preclinical models, we explored a combination of pharmacologic mTOR inhibition and Treg depletion using αCD4 antibody. This is an attractive approach because CD4 depleting antibodies have been studies in patients with peripheral T cell lymphoma (14, 15), Crohn's disease (16), and multiple sclerosis (17, 18), and have a well-established safety profile. However, CD4 depletion removes CD4 effector cells, which are required for initiation of an immune response. Therefore, CD4 depletion was timed to occur after immune priming has taken place. In murine models for RCC and melanoma, mTOR inhibition and CD4 depletion produced a robust cellular immune response that was transferable and effective in controlling subcutaneous tumors as well as lung metastases. The combination treatment produced highly effective memory lymphocytes with robust recall responses. The stimulation of immunological memory due to CD4 depletion was attributed to Treg depletion based on experiments using transgenic models to specifically deplete Tregs ("Treg knock-out") or replace tumor-specific Tregs ("Treg knock-in") following CD4 depletion. Another mechanism contributing to the antitumor response was that Tregs that returned after CD4 depletion were less immunosuppressive than Tregs from mice without CD4 manipulation.

MATERIALS AND METHODS

Mice and tumor cells

Female C57BL/6J, BALB/c mice and Pmel-1 mice, 6–8 week old, were purchased from Jackson laboratory (Bar Harbor, ME) and housed under pathogen-free conditions. FoxP3-GFP mice were a generous gift from Dr. Vijay Kuchroo (Harvard, Boston). DEREG (DEpletion of REGulatory T cells) transgenic mice was generated and described by T.S. (19). All experiments involving animals were in compliance with federal and state standards, which include the federal Animal Welfare Act and the NIH guide for the care and use of laboratory animals.

Human gp100 transduced B 16 cells (B16-gp100) were kindly provided by Dr. Alexander Rakhmilevich from University of Wisconsin-Madison. RENCA, a murine RCC line, was a gift from Dr. Arie Belldegrum (University of California, Los Angeles). All cells were periodically authenticated by morphologic and histologic inspection, and animal grafting for assessing their ability to grow and metastasize. Cells were annually tested for mycoplasma using Myco Alert kit (lonza, Allendale NJ). The cells were maintained in DMEM or RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies, Grand Island, NY), 2 mmol/L of L-glutamine, 100 units/mL of penicillin, and 100 μg/mL of streptomycin.

Mouse tumors were generated by subcutaneously injecting $2x10^5$ cells into the flank. Tumor diameter was measured with calipers twice a week and tumor volume was calculated (shortest diameter^2 × longest diameter/2). In the lung metastasis model, tumor cells were injected intravenously through the tail vein. Lung metastases were counted using a dissection microscope.

Antibodies and reagents

The following monoclonal antibodies (mAb), with or without a fluorescent conjugate, were obtained from Biolegend (San Diego): anti-CD4 (GK 1.5 and RM4-5), anti-CD8 (53-6.7), anti-CD16/CD32(9.3), anti-CD90.1 (OX-7), anti-CD11c (N418), anti-Bcl2 (BCL/10C4), anti-T-bet (4B10), anti-CD62L (MEL-14), anti-CD279 (PD-1,29F.1A12), anti-FoxP3 (FJK-16s), anti-IFN-γ (XMG1.2), anti- IL-2 (JES6.5H4), anti-IL-4 (11B11), IL-17A (eBio1787). CellTrace 5-(and 6-)carboxyfluorescein diacetate succinimidyl ester (CFSE) cell proliferation kit was purchased from Invitrogen (Eugene, OR). CD4 (GK1.5) and CD8 (2.43) antibodies for T cells depletion were purchased from BioXcell (West Lebanon, NH). Temsirolimus was purchased from LC Laboratory (Woburn,MA).

T cell enrichment and Treg sorting

Mouse spleen and lymph nodes were collected and processed into single-cell suspensions. CD8 and CD4 T-cells were negatively enriched using mouse CD8 or CD4 recovery column kits (Cedarlane Labs, Burlington, NC). Purity of CD8 and CD4 cells after negative selection was greater than 85%. FoxP3-GFP cells or antibody stained CD4+CD25+ cells were sorted by MoFlo Cell Sorter (Fort Collins, CO).

Preparation of DCs and T cell stimulation

DC preparation has been described (2). To prepare DC vaccine for treatment of mice, DCs were pulsed with tumor cell lysate and activated with 10ug/ml CpG . DCs were subcutaneously injected into mouse. For in vitro activation of Pmel-1 cells, DC was pulsed with 10ng/ml mouse gp100 peptide (amino acids 25-33, which is presented by H2-Db class I molecules, Alpha Diagnostic International, San Antonio, TX) and activated with 10ug/ml CpG for 2 hours. DC was washed with PBS, and co-cultured with CFSE labeled Pmel-1 cells. Pmel-1 cells proliferation was analyzed by FACscan.

Adoptive transfer, CD4 cells depletion, and mTOR inhibition

Pmel-1 lymphocytes were isolated from lymph nodes and spleen of naïve Pmel-1 mice. CD8 lymphocytes were enriched by negative selection using Cedarlane purification column. At least 85% of the resulting cells were CD8+. $5x10^5$ cells were transferred into B57BL/6 mice. The day after adoptive transfer, mice received tumor lysate pulsed DC vaccine. To deplete CD4 cells, αCD4 was administered approximately 7 and 9 days later; mice were inject ip with 250ug of CD4 mAb (clone GK1.5). To deplete CD8 cells, mice received 250ug of CD8 mAb (clone 2.43). To deplete FoxP3 cells in DEREG mice, 5 μg DT was injected. Flow cytometry was used to confirm depletion of target cells. For mTor inhibitor treatment, 15 μg temsirolimus was injected i.p. each day for 2 weeks. Flow cytometry was used to analyze memory cells and Treg cells.

CTL assay

The in vivo CTL assay has been described (2). For the in vitro CTL, fresh splenocytes were cultured in the presence of IL2 (40IU/ml, eBioscience, San Diego, CA), tumor lysate and H-2K-restricted CA9 peptide(20) for 5 days. Live cells were isolated with the Lympholyte® Media (Cedarlane Labs, Burlington, NC) and used as effector cells. To prepare target cells, RENCA cells were treated with IFN-γ (20ng/ml, eBioscience) for 24 hours and labeled with CFSE (0.5μmol/L). Effector and target cells were co-cultured at a ratio of 50:1 at 37°C for 12 hours. Cells were stained with 7-AAD and PE labelled annexin V (eBioscience) on ice for 20 min and analysed by flow cytometry for percent of 7-AAD+annexin V- cells that were CFSE+.

Statistics

Differences in tumor growth were assessed using repeating measure ANOVA. Statistical differences were evaluated by two-tailed student's T test. All statistical analyzes were performed using Stata 8.0 (StataCorp, College Station, Texas). *P* values < 0.05 were considered significant.

RESULTS

mTOR inhibition enhances anti-tumor immunity.

In animal models, pharmacologic mTOR inhibition can enhance the formation of immune memory, which can help clear infections (1, 4) and decrease tumor growth (2, 3). This was a surprising finding because mTOR inhibitors are used to suppress the immune system in patients who have had solid organ transplants. Temsirolimus is a rapamycin analog and one of the first mTOR inhibitors approved by the US, Food and Drug Administration (FDA) as a cancer treatment. In our preclinical model, mTOR inhibition with temsirolimus enhanced the antitumor immunity of tumor lysate-pulsed DCs (referred to here as DC vaccine) (Figure 1A). Temsirolimus can directly inhibit the growth of some tumors(2), therefore a tumor prevention study was performed to assess the immune effects of temsirolimus. By administering DC vaccine and temsirolimus 13 days prior to tumor challenge, there is no possibility for a direct antitumor effect, and any decrease in tumor growth can be attributed to immune stimulation. Administering DC vaccine alone decreased growth of B16 tumor cells in mice, however, most mice eventually died due to tumor growth. In contrast, the combination of DC vaccine and temsirolimus resulted in 100% survival and completely prevented the growth of B16 tumor cells.

To assess the immune effect of temsirolimus on specific CD8 lymphocytes, Thy1.1 Pmel-1 lymphocytes were adoptively transferred into Thy1.2 B6 mice (Figure 1B & C). Pmel-1 transgenic mice carry a rearranged T cell receptor that recognizes a gp100 epitope (amino acids 25-33) presented by H2-Db MHC class I molecules. Lymphocytes were harvested from B6 mice after they were treated with DC vaccine and temsirolimus. Temsirolimus had both immune stimulating and immune suppressing effects when administered with the DC vaccine. Temsirolimus slightly decreased the percent of CD8 cells that were Pmel-1 lymphocytes (p-value 0.08), however, Pmel-1 lymphocytes had increased expression of Eomes, which is an

early marker for memory cell formation (3, 21). Potentially immune suppressive effects included a decrease in Tbet expression in Pmel-1 lymphocytes and increase in Tregs. These observations were largely mirrored by *in vitro* mixed lymphocyte culture studies (Figure 1D & E). In the *in vitro* studies, temsirolimus significantly decreased the proliferation of Pmel-1 lymphocytes induced by the DC vaccine.

CD4 depletion enhanced the antitumor effect of mTor inhibition.

Temsirolimus produced a net antitumor immune response despite an increase in Tregs. Furthermore, the presence of tumor itself increased Tregs (Supplemental Figure 1). Therefore, we hypothesized that the antitumor immunity induced by mTOR inhibition can be further enhanced by targeting Tregs. Currently there is no clinical strategy to selectively remove Tregs; however, it is feasible to deplete all CD4 lymphocytes. However, CD4 effector cells are required for immune activation. Therefore, in mouse models, CD4 lymphocytes were depleted with αCD4 IgG2b antibody (αCD4) after immune stimulation by the implanted tumor (Figure 2).

We tested this approach in a second model of renal cell carcinoma, another classically immune sensitive tumor. In a tumor treatment model, palpable RENCA tumors were established in Balb/c mice (Figure 2). Temsirolimus has been shown to have direct cytostatic growth inhibition of RENCA tumor cells *in vitro* (2), and as expected temsirolimus alone was effective in decreasing tumor growth in our mouse model (Figure 2A). However, when temsirolimus was stopped, the tumor started growing again (data not shown). Addition of αCD4 to temsirolimus treatment further decreased tumor growth and was even curative while αCD4 alone had no effect. It is interesting to note that the combination of αCD4 and temsirolimus decreased tumor growth even when no cancer vaccine was used and the implanted tumor was the only source of specific immune stimulation.

In the same experiment, lymphocytes were harvested on day 45 and assessed for tumor-specific IFN-γ response (Figure 2B) and CTL killing (Figure 2C). In tumor-bearing mice that received no treatment, there was no IFN-γ or killing response. Treatment with either αCD4 or temsirolimus produced some IFN-γ or killing response; however, the combination treatment produced the largest responses. To characterize the CD4 lymphocyte depletion in response to αCD4, naïve mice were treated with a single dose of αCD4. Nearly all CD4 cells were depleted from the peripheral blood, spleen and lymph nodes by the next day (Figure 2D) while CD8 cells were preserved (Figure 2E). Importantly, FoxP3+CD4+ cells were depleted and remained low even 10 days following administration of αCD4 (Figure 2F). A single dose of αCD4 reduced the population of all CD4 subsets (Supplemental Figure 2).

There are known differences in the immune system of Balb/c and B6 mice. (22) However, a similar antitumor effect was observed with our proposed therapy in B6 mice with established B16 tumors (Supplemental Figure 3). Unlike RENCA, the B16 tumors are not directly inhibited by temsirolimus.(2) Therefore, antitumor effects seen in the presence of mTOR inhibition are likely produced by the immune system. Another consideration is that B16 tumors grow very rapidly, and most mice require euthanasia within three weeks of tumor implantation. Therefore, DC vaccines were used in our tumor treatment models to decrease B16 growth rates and give our treatments sufficient time to stimulate the immune system.

Combination of CD4 depletion and temsirolimus generated anti-tumor immunity that was dependent on memory CD8 cells.

Since temsirolimus can directly inhibit RENCA cells, it was important to establish that the combination of αCD4 and temsirolimus was generating an effective antitumor immunity dependent on CD8 lymphocytes. Balb/c mice bearing RENCA tumors were treated with temsirolimus alone for 10 days and then challenged with a second RENCA tumor (Figure 3A).

Mice injected with αCD8 antibody (αCD8) to deplete CD8 effectors cells had increased growth of the second RENCA tumor, indicating that even temsirolimus alone works at least in part by stimulating an immune response. The combination of temsirolimus and αCD4 completely prevented the growth of second RENCA tumors; however, αCD8 removed the antitumor effect on the second tumors, indicating the importance of cellular immunity to tumor control (Figure 3B).

To further establish the role of the immune system and test our proposed treatment in a more aggressive tumor model, we assessed whether antitumor immunity can be transferred to prevent growth of metastatic lung deposits. The combination of αCD4 and temsirolimus was used to treat established, subcutaneous RENCA tumors (Figure 3C). Lymphocytes from these mice were adoptively transferred to naïve mice, which were challenged intravenously with RENCA cells. The combination treatment significantly decreased the establishment and growth of lung deposits (Figure 3C) as quantified by comparing lung weights (Figure 3D) and counting lung deposits (Figure 3E). Specific IFNγ expression in CD8 lymphocytes was increased in the combination therapy group (Figure 3F). Therefore, memory cells were successfully transferred into naïve mice, where they helped control tumor growth.

Combination of CD4 depletion and temsirolimus treatment enhanced function of CD8 memory cells.

An important mechanism through which temsirolimus inhibits tumor growth is to enhance the quality of specific CD8 memory (1, 2). Therefore, we characterized the quality of CD8 memory cells with the goal of assessing whether αCD4 further enhances the specific CD8 memory formed in the presence of mTOR inhibition. We used a model where DC vaccines stimulated an immune response rather than the tumor itself since the duration of experiments with tumor-bearing mice is limited by rapid tumor growth in the control groups. By using a DC vaccine,

long-term memory can be assessed, including recall responses. Thy1.1 Pmel-1 lymphocytes were adoptively transferred into B6 mice, which were then challenged with B16-DC vaccine and treated with αCD4 and temsirolimus (Figure 4A).

To assess memory cells, splenocytes were harvest before (Figure 4B) or after (Figure 4C) rechallenging mice with DC vaccine on day 46. Immediately prior to rechallenge, there was no significant difference in percent of Pmel-1 lymphocytes in the experimental groups (Figure 4B). However, the CD8 lymphocytes from mice treated with both αCD4 and temsirolimus had significantly higher expression of memory markers Eomes and BCL2. The CD8 lymphocytes from this group had significantly higher expression of CD62L, which is a marker for highly effective central memory cells. Consistent with high quality memory cells, following rechallenge with DC vaccine, the Pmel-1 cells in the combination treatment group had the greatest expansion and CD8 cells had the highest Tbet and IL2 expression (Figure 4C). Interestingly, even after rechallenge, the expanded CD8 cells from the combination treatment group had the highest expression of Eomes.

Others have reported that Tregs are necessary, during immune priming, for CD8 memory formation (23, 24). Therefore, in our treatment models, Tregs were depleted at least 6 days after primary immune stimulation. However, we verified the importance of having CD4 cells during immune priming. When CD4 depletion was performed prior to immune priming, CD8 memory formation was poor, as indicated by a weak tumor-specific CD8 expansion after stimulation of memory cells and low Eomes expression (Supplemental Figure 4).

Depleting or Replacing Foxp3 Treg cells alter CTL function *in vivo*

Our original hypothesis was that depletion of Tregs normally induced by temsirolimus will enhance antitumor immunity. We selected CD4 depletion as a strategy for depleting Tregs since CD4 depletion is feasible in patients. However, we wanted to test whether the effect of

CD4 depletion can be directly attributed to Tregs depletion. Therefore we used DEREG (DEpletion of REGulatory T cells) transgenic mice, which carry a DTR-eGFP transgene under the control of Foxp3 promoter, allowing specific depletion of Tregs by administering diphtheria toxin (DT) (19). In an experiment analogous to one shown in figure 2, DT was administered on days 6 and 10, in place of αCD4 (Figure 5A). The immune system was stimulated with DC vaccine and specific immune memory was assessed on day 35 by *in vivo* CTL and IFN-γ staining. DT administration removed nearly all CD4+FoxP3+ lymphocytes (Figure 5B). Specific killing and IFN-γ staining significantly increased in mice treated with DT and temsirolimus when compared to control groups (Figure 5C & D). Therefore, removing Tregs had a similar immune effect to CD4 depletion.

To fully establish Treg depletion as the underlying mechanism for immune stimulation following CD4 depletion, Tregs were replaced after CD4 depletion (Figure 6A). Mice treated with αCD4 and temsirolimus developed the best specific immune memory as assessed by *in vivo* CTL (Figure 6B). However, when Tregs from mice treated with DC vaccine were adoptively transferred, specific killing and CD8 lymphocyte IFN-γ response decreased to that of control mice that only received the DC vaccine (Figure 6B & 6C). These experiments confirm that with αCD4 it is the Treg depletion that enhances specific immune memory formation.

Following CD4 depletion, Treg population that eventually recovers is less immunosuppressive.

Following treatment with DC vaccine, αCD4, and temsirolimus, the Treg population eventually recovers (Figure 7A). Between experimental groups, the differences in absolute number of Tregs in the spleen were not statistically significant. However, the treatments may have had a long-term effect on Treg function. Therefore, we assessed the immunosuppressive function of the recovered Tregs. CD4 lymphocytes were sorted based on CD25 status (Figure 7B). The

vast majority of the CD4+CD25+ cells were FoxP3 positive and were considered Tregs, and the vast majority of CD4+CD25- cells were FoxP3 negative and were considered CD4 effector cells. In functional studies, control CD4+CD25+ cells suppressed the proliferation of CD8 lymphocytes. However, CD4+CD25+ that recovered after CD4 depletion were less immunosuppressive, possibly because they were less likely to be tumor-specific Tregs (Figure 7D). Interestingly, following CD4 depletion, the recovered CD4 effector cells were also less effective as indicated by lower IFNγ and IL4 production (Figure 7C). It is possible that both CD4 effector cells and Tregs were less likely to be tumor-specific.

DISCUSSION

Immunotherapeutic approaches have proven effective for the treatment of solid tumors. The FDA approved sipuleucel-T, which became the first commercially available cancer vaccine for the treatment of a solid tumor (25). Ipilimumab, a monoclonal antibody targeting CTLA4, was more recently approved for the treatment of melanoma (26). Immune checkpoint inhibitors that target CTLA4 and PD-1 are being actively investigated in a large number of clinical trials for various malignancies. There have always been hints that immune-based therapies can even be curative in subsets of patients with metastatic disease (27), but recent advances in immunotherapy reaffirm that durable complete responses are possible (28). Therefore, immunotherapy is one of the most promising approaches to cancer therapy.

A hallmark of durable immune responses is the generation of immune memory. The mTOR pathway has emerged as a critical determinant of immune memory (1-3). We confirmed prior observations that pharmacologic mTOR inhibition with temsirolimus can enhance the efficacy of adoptive immunotherapy (Figure 1). Temsirolimus enhanced the expression of CD8 lymphocyte markers associated with memory formation, both *in vivo* and *in vitro*. However, it also increased the proportion of T cells expressing FoxP3. This was an expected finding since the canonical mTOR inhibitor, rapamycin, is routinely used in the clinic as an immune suppressant, and its primarily mode of action is believed to be through enhanced Treg activity (29, 30).

We reasoned that a combination therapy that includes a strategy to control Tregs would further enhance antitumor immunity. We elected to use a depleting αCD4 antibody because of its potential for clinical translation. The combination of αCD4 and temsirolimus was highly effective in controlling established RENCA tumors even when a cancer-specific vaccine was not used (Figure 2). Temsirolimus is known to directly inhibit the growth of RENCA cells (2); therefore, we assessed the immune contribution to the antitumor effect. By depleting CD8 cells, antitumor

activity was shown to be dependent on CD8 cells (Figure 3). Further confirmation of immune stimulation was provided by transferring CD8 lymphocytes from treated mice to naïve mice. In a very aggressive tumor model, the transferred lymphocytes were effective in controlling growth of metastatic deposits.

Temsirolimus enhanced CD8 memory formation. Therefore, we assessed memory formation with the combination of temsirolimus and αCD4 (Figure 4). The combination therapy produced CD8 lymphocytes with the strongest memory phenotype, capable of rapidly expanding in response to a repeat antigen challenge. Although Tregs have been described as a barrier to formation of antitumor memory (31), other recent studies indicate that Tregs are required during immune priming to generate high-avidity primary responders and functional CD8 memory (23, 24). In our model, Tregs were present during immune priming since CD4 depletion was initiated at least 6 days after immune stimulation.

CD4 depletion was also timed to allow CD4 effector cells to help prime CD8 cells and contribute to memory lymphocyte formation (Figures 2-4). During immune priming, CD4 activity is dominated by helper function. Although it has been reported that CD4 cells are not required for priming, they are required for effective CD8 memory formation (32-34). CD8 memory cells formed in the absence of CD4 cells had an exhausted phenotype and increased PD-1 expression (32, 34). In our study, the CD8 memory cells formed in the absence of CD4 were less capable of responding to a second challenge and had lower Eomes expression (Supplemental Figure 4). Following immune priming, CD4 activity is dominated by regulatory function. Therefore, we and others have shown that CD4 depletion at this point leads to enhanced formation of both central and effector CD8 memory (35) and enhanced tumor control (35, 36).

Prior studies have assumed that the effects of CD4 depletion were due to Treg depletion. In this study, we use two separate experiments to empirically identify Treg depletion as the underlying mechanism: we selectively depleted FoxP3 expressing cells ("knock-down") or replaced FoxP3 expressing cells following CD4 depletion ("knock-in") (Figures 5,6). These experiments clearly identify Treg depletion as the mechanism accounting for enhanced immunity following CD4 depletion. Interestingly, intratumor Treg depletion has been identified at the primary mode of action for ipilimumab (37), which has been proven effective in a phase III trial of melanoma (38). Therefore, an intriguing possibility is that CD4 depletion may produce a similar immune effect in patients as ipilimumab.

Our study provides a preclinical rationale for CD4 depletion in patients. Multiple clinical trials have already documented the safety of CD4 depletion in humans (14, 15, 17, 39-44). Near complete CD4 depletion was achieved in several trials of chimeric αCD4 antibodies for refractory cutaneous T-cell lymphoma, and no serious infections or other dose-related toxicities were noted (14, 15, 44). A CD4 depleting chimeric monoclonal antibody was evaluated in two separate randomized, double-blind, placebo-controlled studies for rheumatoid arthritis (41, 43). Although CD4 depletion did not have a therapeutic effect for rheumatoid arthritis, there were no significant adverse event, and no patient suffered from opportunistic infections. Even long-term CD4 depletion appears to be safe since some patients in one of the trials were treated with αCD4 for more than 9 months (43). After completing CD4-depleting treatment, patients have been followed for safety: 24 patients were followed for 30 months and no opportunistic infections or other significant adverse effects were seen (40). Additional, strong support for the safety of CD4 depletion comes from a clinical trial where CD4 depletion was added to a standard immunosuppressive regiment in heart transplant patients. These are patients where standard therapy already puts them at high risk for opportunistic infections. Patients in the CD4

depletion group actually had fewer episodes of infection than patients receiving standard immunosuppression (39).

Several studies have tried to explain why CD4 depletion had no therapeutic effect for autoimmune disorders. In multiple clinical trials, αCD4 preferentially depleted naïve CD4 cells and tended to preserve memory CD4 cells (17, 45, 46). One study estimated that unprimed CD4 cells were 3 times more sensitive to depletion than primed CD4 cells (45). This feature is desirable for cancer immunotherapy. In addition, CD4 depletion decreased the number of IL4-producing T helper 2 (Th2) cells while the number of IFNγ-producing T helper 1 (Th1) cells remained stable, thus significantly increasing the Th1/Th2 ratio. In our animal model, we also noted that the percent of IL2 producing Th1 cells increased following CD4 depletion because of larger drops in number of Th2 and Th17 cells (Supplemental Figure 3). Therefore, the persistence of Th1 memory cells, along with CD8 memory cells, may explain the lack of benefit when treating chronic autoimmune disease. However, previously primed memory cells are beneficial for cancer immunotherapy where Th1 and CD8 memory cells contribute to antitumor immunity.

Another important observation in preclinical tumor models is that even partial CD4 depletion may be effective for enhancing the efficacy of cancer vaccines (47). Following CD4 depletion, CD4 counts and Tregs recover. However, the Tregs that recover were less immunosuppressive (Figure 7). Antigen-specific Tregs have been reported to suppress antitumor immunity (48) and it is possible that the recovered Tregs may be less likely to be tumor-specific.

The possibility of combining a CD4 antibody with mTOR inhibitors, which are already commercially available to treat cancers, is attractive. The tumor itself may provide sufficient antigen stimulation and additional adoptive immunotherapy may not be needed. The combination therapy appears to enhance immune memory and remove Tregs. A clear

understanding of the mechanism-of-action of the proposed therapy helps bolster the rationale for further clinical investigation.

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FIGURE LEGENDS

Figure 1. **There are both immune stimulating and inhibiting effects of mTOR inhibition; however the net effect is enhanced anti-tumor immunity.**
A. Experimental scheme for a melanoma tumor prevention model: mice (n=5 per group) received tumor lysate-pulsed DC vaccine on days -30 and -23, and temsirolimus was injected intraperitoneally daily on days -23 to -13. B16 tumor cells were injected subcutaneously in the flank on day 0. B16 tumor growth and survival curves are shown.
B. Experimental scheme to characterize lymphocytes following treatment with DC vaccine and temsirolimus: Thy1.1 Pmel-1 lymphocytes were adoptively transfer into Thy1.2 B6 mice, which received tumor lysate-pulsed DC vaccine on day -6, and daily temsirolimus for 5 days. Splenocytes were harvested on day 0, and stained for CD8, Thy1.1, Tbet, Eomes and CD4/FoxP3 and analyzed by flow cytometry. Representative results are shown.
C. Summary data (n=5) are provided for Figure 1B.
D. Lymphocytes were characterized with *in vitro* mixed cultures using pmel-1 lymphocytes and tumor lysate-pulsed, CpG activated DCs treated with temsirolimus for 48 hours. Lymphocytes were stained for CD8, Thy1.1, Tbet, Eomes and CD4/FoxP3 and analyzed by flow cytometry. Representative results are shown.
E. Summary data (n=3) are provided for Figure 1D.
All results are representative of at least duplicate experiments. Histograms provide mean $\pm$ SEM. *p<0.05, **p<0.01,***p<0.005

Figure 2. **CD4 depletion enhanced the antitumor effect of mTor inhibition.**
A. RENCA-CA9 tumor cells were implanted into Balb/C mice (n=5 per group) on day 0. CD4 lymphocytes were depleted with αCD4 antibody on days 6 and 10. Mice were treated with daily temsirolimus on days 14 to 34. Tumor growth was monitored. Results are representative of triplicate experiments.
B. In the same experiment, lymphocytes were harvested on day 45, restimulated with CA9 peptide, and stained for CD8 and IFNγ. Results are representative of duplicate experiments.
C. For the *in vitro* CTL assay, splenocytes were harvest on day 45 and cultured with IL2, RENCA lysate and CA9 peptide. Target cells were prepared by labeling RENCA cells with CFSE. Effector and target cells were co-cultured at a ratio of 50:1 and analyzed by FACS for the percent of CFSE+ cells that were 7-AAD positive and annexin V negative.
D. Following CD4 depletion, spleen, lymph node and blood were collected on days 0, 1, 10. Lymphocytes were stained for CD4, CD8 and FoxP3 and analyzed by flow cytometry. (c-e) The percentages of CD4 cells in the spleen, lymph node and blood on days 0, 1, and 10 following CD4 depletion are reported.
E. Following CD4 depletion, percentages of splenocytes that are CD4 or CD8 positive are reported, and percent of CD4 cells that are FoxP3 positive is reported.
F. In the same experiment, the absolute numbers of splenocytes positive for CD4, CD8, and CD4/FoxP3 are reported.
Histograms provide mean $\pm$ SEM. *p<0.05, **p<0.01,***p<0.005

Figure 3. **Combination of CD4 depletion and temsirolimus generated anti-tumor immunity that was dependent on memory CD8 cells.**

A. Tumor-bearing mice (n=8) were treated with daily temsirolimus and then rechallenged with RENCA-CA9 35 days after primary tumor implantation. CD8 cells were depleted by injecting αCD8 antibody on day 36. Results are representative of duplicate experiments.
B. Tumor-bearing mice (n=8) treated with temsirolimus and CD4 depletion, and then rechallenged with RENCA-CA9 35 days after primary tumor implantation. CD8 cells were depleted by injecting αCD8 antibody on day 36.
C. - E. Lymphocytes were harvested from mice treated with temsirolimus and CD4 depletion. The lymphoctes were cultured *in vitro* with CA9 peptide and IL2 (10u/ml) for 3 days and then adoptively transferred into naïve B6 mice, which were challenged 24 hrs later with $2x10^5$ RENCA tumor cells injected i.v. Lungs were collected 30 days after the i.v. tumor challenge. Lung weight (d) and number of lung tumor deposits (e) were determined.
F. At the time of lung collection (day 30), lymphocytes were harvested on day 30, restimulated with CA9 peptide and stained for CD8 and IFNγ.
Histograms provide mean ± SEM. IL2, interleukin-2, *p<0.05, **p<0.01,***p<0.005

Figure 4. **Combination of CD4 depletion and temsirolimus treatment enhanced function of CD8 memory cells.**
A. Experimental scheme: Lymphocytes from Thy1.1 Pmel-1 mice were adoptively transferred into Thy1.2 B6 mice, which were stimulated with tumor lysate-pulsed DC vaccine and treated with αCD4 antibody on days 7 and 10, and daily temsirolimus on days 10 to 24. Results are representative of triplicate experiments.
B. Splenocytes (n=3 per group) were harvested one day prior to rechallenging with tumor lysate-pulsed DC vaccine, stained with antibodies, and analyzed by flow cytometry. The percent of total CD8 cells positive for the indicated marker is shown.
C. Splenocytes (n=3 per group) were harvested 4 days after rechallenging with tumor lysate-pulsed DC vaccine, stained with antibodies, and analyzed by flow cytometry. The percent of CD8 cells positive for the indicated marker is shown.
Histograms provide mean ± SEM. *p<0.05, **p<0.01,***p<0.005

Figure 5. **The combination of FoxP3+ Treg depletion and temsirolimus enhanced CTL function *in vivo.***
A. Experimental scheme: DEREG mice received tumor lysate-pulsed DC vaccine (n=5 per group), and were treated intraperitoneally with diphtheria toxin on days 6 and 10, and daily temsirolimus on days 10 to 20. *In vivo* CTL assay was performed on day 35. The mice in the control group received DC vaccine alone.
B. CD4+FoxP3+ cells were assessed by flow cytometry using peripheral lymphocytes obtained before and after treating mice with diphtheria toxin.
C. The *in vivo* CTL results were analyzed by flow cytometry using splenocytes harvested 14 hrs following injection of target cells.
D. On day 35, splenocytes were harvested and stained for CD8 and specific IFN-γ expression.
Histogram provides mean ± SEM. CTL, cytotoxic T lymphocyte, *p<0.05, **p<0.01,***p<0.005

Figure 6. **Adoptive transfer of FoxP3+ Tregs reduced CTL function *in vivo* in mice treated with the combination of CD4 depletion and temsirolimus.**

A. Experimental scheme: B6 received tumor lysate-pulsed DC vaccine, and were treated intraperitoneally with αCD4 antibody on days 6 and 10, and daily temsirolimus on days 14 to 24 (n=6 per group). Tregs, sorted from lymphocytes from GFP-FoxP3 mice that received tumor lysate-pulsed DC vaccine (pooled from 3 mice), were adoptively transferred on day 20 and *in vivo* CTL assay was performed on day 35.

B. The in vivo CTL results were analyzed by flow cytometry using splenocytes harvested 14 hrs following injection of target cells (n=3-6 per group). Results are representative of duplicate experiments.

C. On day 45 mice were re-stimulated with DC vaccine and splenocytes were stained for CD8 and IFN-γ expression.

Histogram provides mean $\pm$ SEM. *p<0.05, **p<0.01,***p<0.005

Figure 7. **After treatment with CD4 depletion, Treg population that recovers is less immunosuppressive.**

A. Mice (n=5 per group) were treated using the experimental scheme outlined in Figure 4A. On day 45, splenocytes were examined by flow cytometry for CD4+FoxP3+ cells.

B. The splenocytes that recovered after DC vaccine with or without CD4 depletion were used to enrich for CD4+ cells, which were then sorted by CD25 status. For the resulting groups, representative flow cytometry for CD4+CD25+ status and CD4+CD25- status are shown.

C. CD4+CD25- cells were co-cultured with DCs pulsed with B16 tumor lysate. The CD4+CD25- cells were analyzed by flow cytometry for CD4-IFNγ or IL4 expression.

D. CD4+CD25+ cells were co-cultured with DCs pulsed with B16 tumor lysate and CD8 cells from mice immunized with DCs pulsed with B16 tumor lysate. CD8 cell proliferation was monitored by flow cytometry using a CFSE dilution assay.

Histograms provide mean $\pm$ SEM. *p<0.05, **p<0.01,***p<0.005

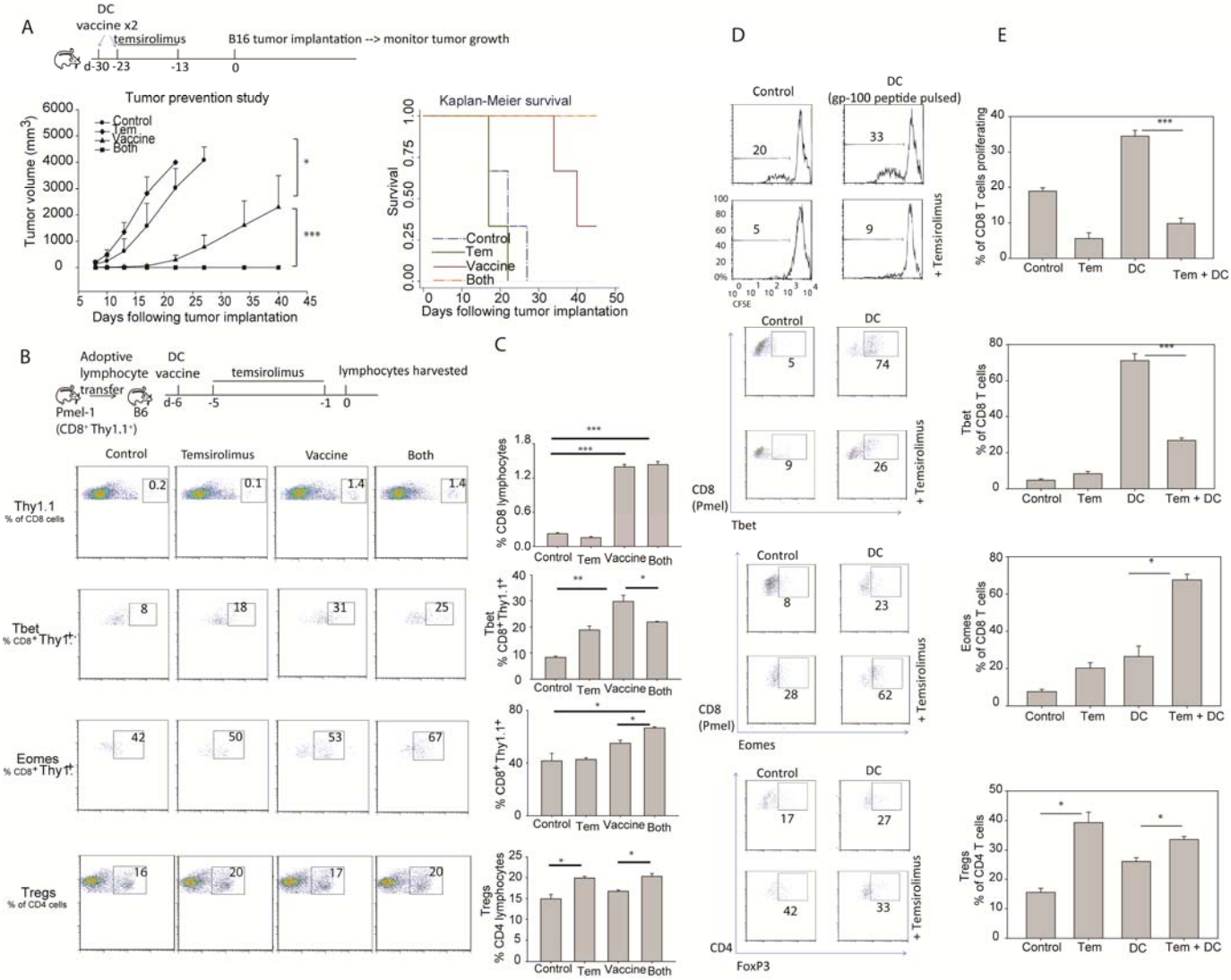
A
DC
vaccine x2
temsirolimus
B16 tumor implantation --> monitor tumor growth
d-30 -23 -13 0
Tumor prevention study
Tumor volume (mm3)
Control
Tem
Vaccine
Both
Days following tumor implantation
Kaplan-Meier survival
Survival
Control
Tem
Vaccine
Both
Days following tumor implantation
B
Adoptive lymphocyte transfer
DC vaccine
temsirolimus
lymphocytes harvested
Pmel-1 (CD8+ Thy1.1+)
B6
d-6 -5 -1 0
Control
Temsirolimus
Vaccine
Both
Thy1.1 % of CD8 cells
Tbet % CD8+ Thy1+
Eomes % CD8+ Thy1+
Tregs % of CD4 cells
C
% CD8 lymphocytes
% CD8+Thy1.1+
Tbet % CD8+Thy1.1+
% CD8+Thy1.1+
Tregs % CD4 lymphocytes
Control Tem Vaccine Both
D
Control
DC (gp-100 peptide pulsed)
+ Temsirolimus
CFSE
CD8 (Pmel)
Tbet
Eomes
CD4
FoxP3
E
% of CD8 T cells proliferating
Tbet % of CD8 T cells
Eomes % of CD8 T cells
Tregs % of CD4 T cells
Control Tem DC Tem + DC

Figure 1

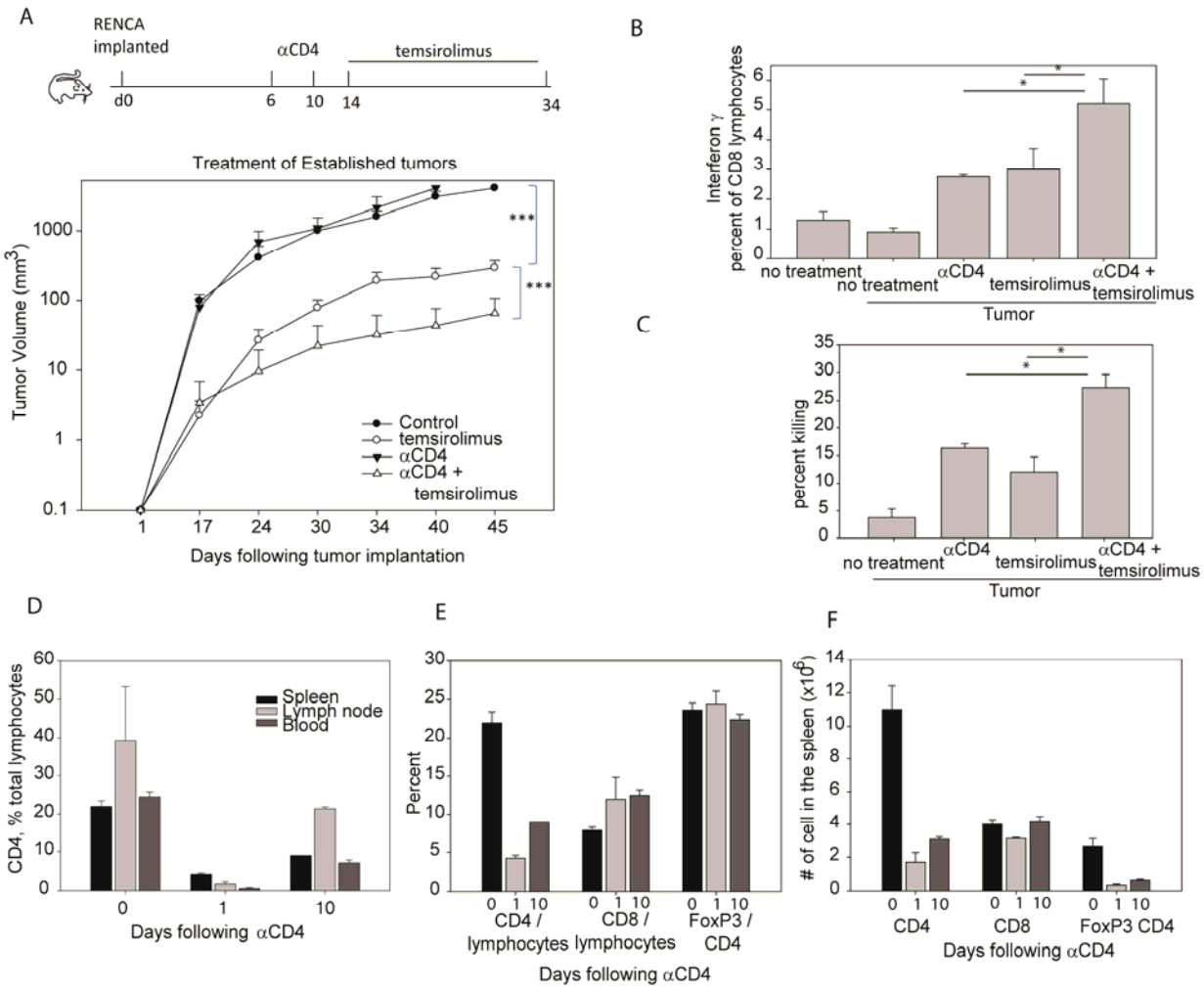
A
RENCA implanted
αCD4
temsirolimus
d0
6
10
14
34
Treatment of Established tumors
Tumor Volume (mm3)
1000
100
10
1
0.1
***
***
Control
temsirolimus
αCD4
αCD4 + temsirolimus
1
17
24
30
34
40
45
Days following tumor implantation
B
Interferon γ percent of CD8 lymphocytes
no treatment
αCD4
temsirolimus
αCD4 + temsirolimus
Tumor
C
percent killing
no treatment
αCD4
temsirolimus
αCD4 + temsirolimus
Tumor
D
CD4, % total lymphocytes
Spleen
Lymph node
Blood
Days following αCD4
E
Percent
CD4 / lymphocytes
CD8 / lymphocytes
FoxP3 / CD4
Days following αCD4
F
# of cell in the spleen (x10^6)
CD4
CD8
FoxP3 CD4
Days following αCD4

Figure 2

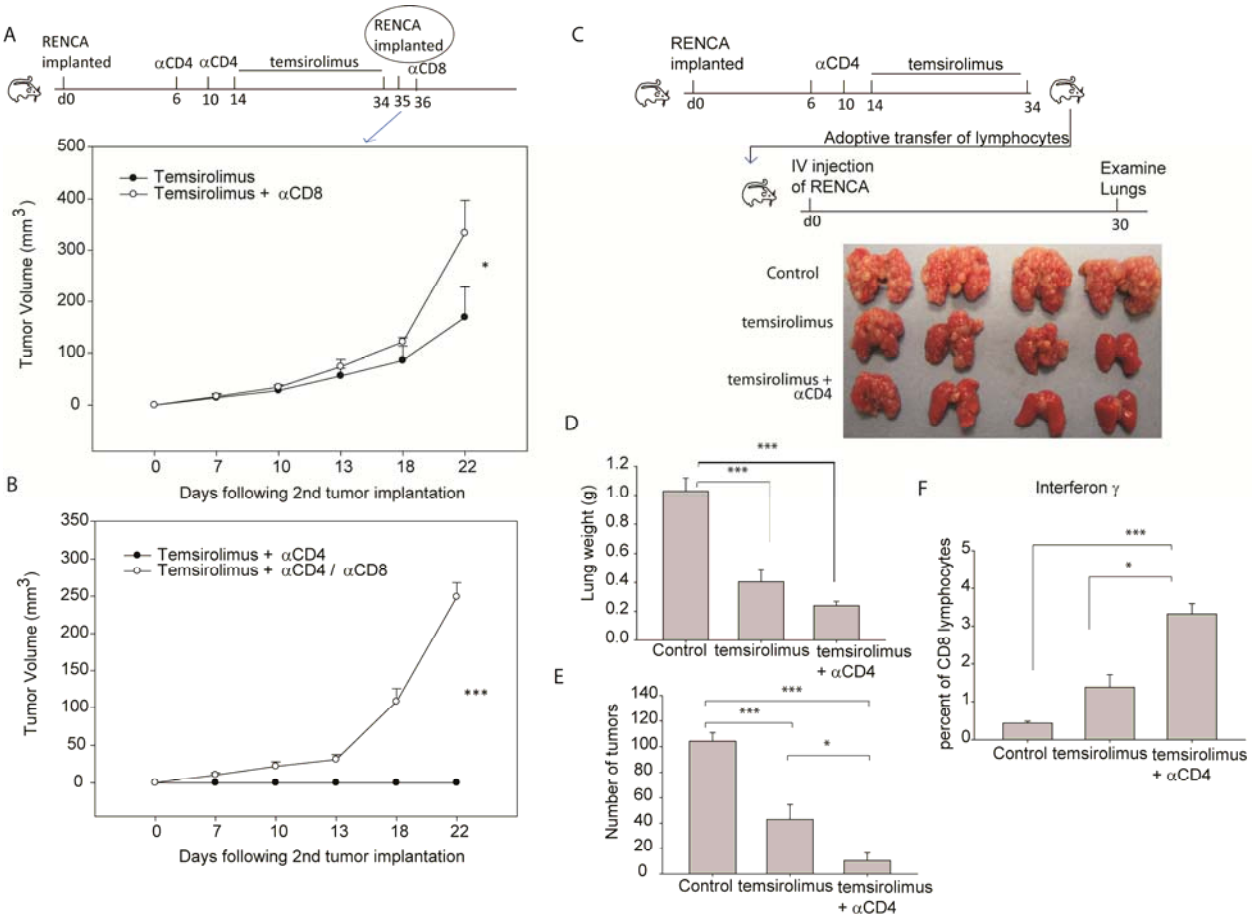
A
RENCA implanted
αCD4 αCD4
temsirolimus
RENCA implanted
αCD8
d0
6
10
14
34
35
36
Temsirolimus
Temsirolimus + αCD8
Tumor Volume (mm3)
Days following 2nd tumor implantation
B
Temsirolimus + αCD4
Temsirolimus + αCD4 / αCD8
Tumor Volume (mm3)
Days following 2nd tumor implantation
C
RENCA implanted
αCD4
temsirolimus
Adoptive transfer of lymphocytes
IV injection of RENCA
Examine Lungs
Control
temsirolimus
temsirolimus + αCD4
D
Lung weight (g)
Control
temsirolimus
temsirolimus + αCD4
E
Number of tumors
Control
temsirolimus
temsirolimus + αCD4
F
Interferon γ
percent of CD8 lymphocytes
Control
temsirolimus
temsirolimus + αCD4

Figure 3

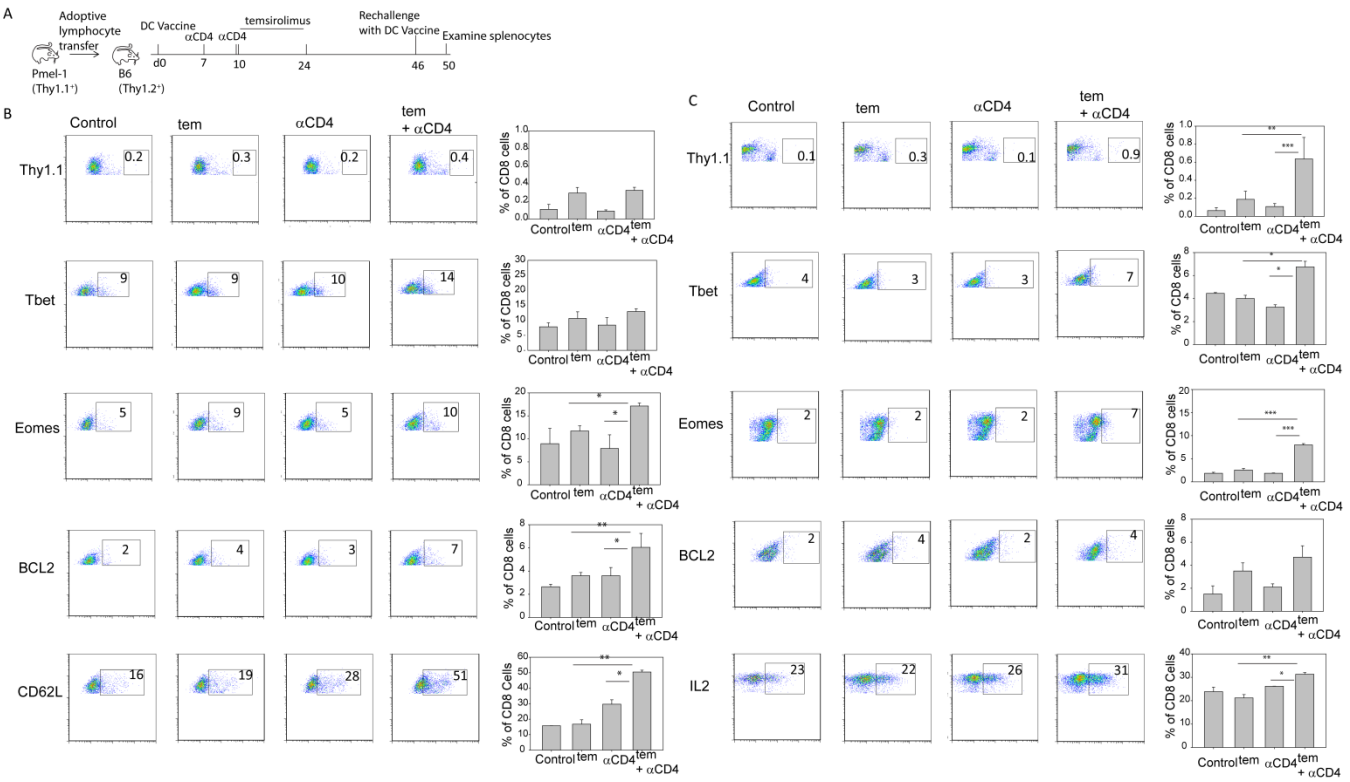
A
Adoptive lymphocyte transfer
DC Vaccine
αCD4 αCD4
temsirolimus
Rechallenge with DC Vaccine
Examine splenocytes
Pmel-1 (Thy1.1+)
B6 (Thy1.2+)
d0 7 10 24 46 50
B
Control
tem
αCD4
tem + αCD4
Thy1.1
Tbet
Eomes
BCL2
CD62L
% of CD8 cells
C
IL2

Figure 4

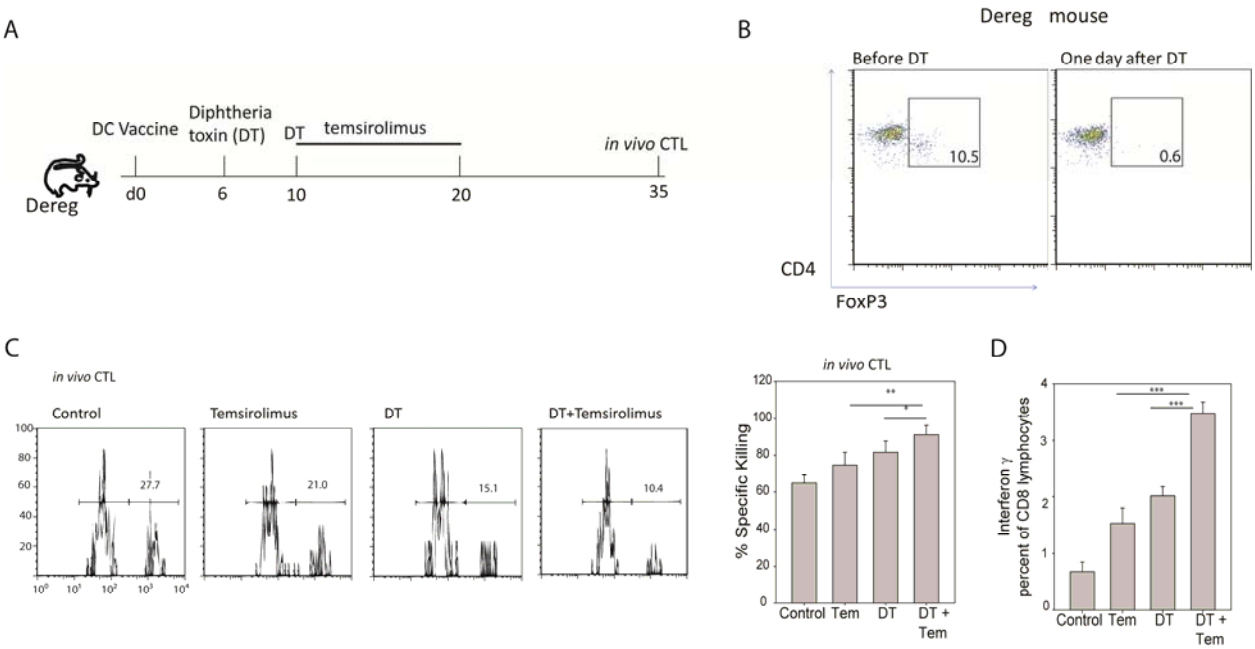
A
DC Vaccine
Diphtheria toxin (DT)
DT
temsirolimus
in vivo CTL
Dereg
d0
6
10
20
35
B
Dereg mouse
Before DT
One day after DT
10.5
0.6
CD4
FoxP3
C
in vivo CTL
Control
Temsirolimus
DT
DT+Temsirolimus
27.7
21.0
15.1
10.4
in vivo CTL
% Specific Killing
Control
Tem
DT
DT + Tem
D
Interferon γ percent of CD8 lymphocytes
Control
Tem
DT
DT + Tem

Figure 5

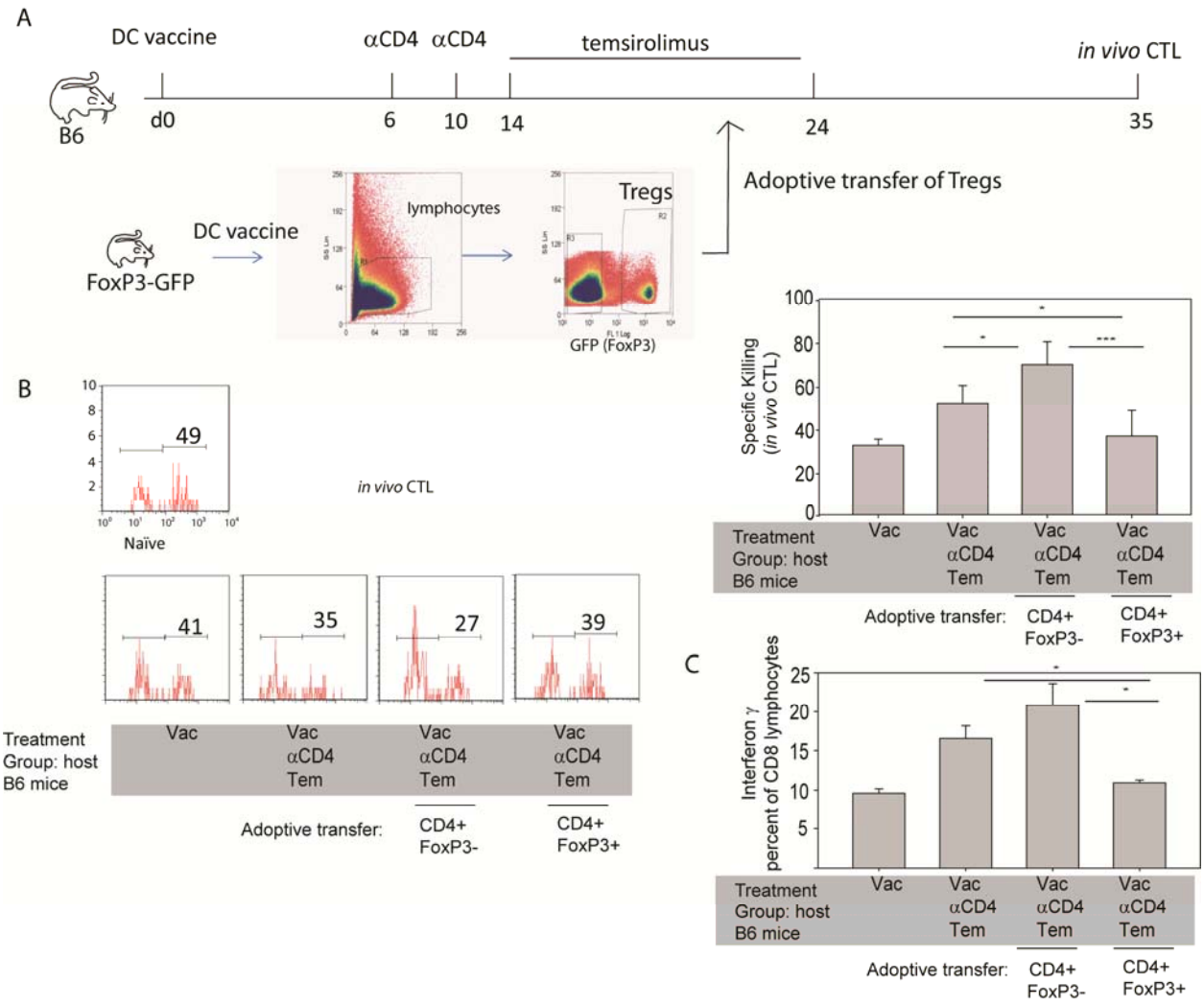
A
DC vaccine
αCD4 αCD4
temsirolimus
in vivo CTL
B6
d0
6
10
14
24
35
Adoptive transfer of Tregs
DC vaccine
FoxP3-GFP
lymphocytes
Tregs
GFP (FoxP3)
B
49
Naïve
in vivo CTL
41
35
27
39
Treatment Group: host B6 mice
Vac
Vac αCD4 Tem
Vac αCD4 Tem
Vac αCD4 Tem
Adoptive transfer:
CD4+ FoxP3-
CD4+ FoxP3+
Specific Killing (in vivo CTL)
C
Interferon γ percent of CD8 lymphocytes

Figure 6

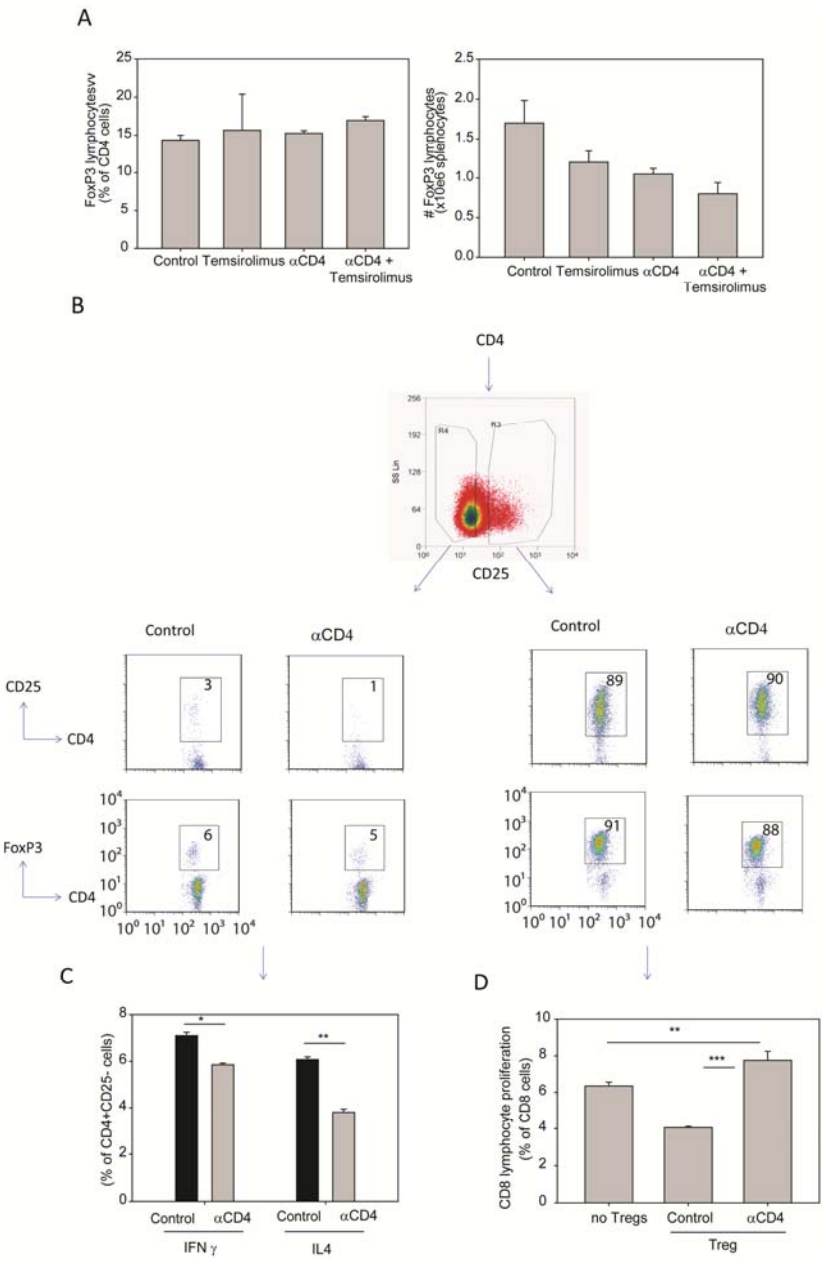
A
FoxP3 lymphocytesvv (% of CD4 cells)
Control Temsirolimus αCD4 αCD4 + Temsirolimus
FoxP3 lymphocytes (X10e6 splenocytes)
Control Temsirolimus αCD4 αCD4 + Temsirolimus
B
CD4
CD25
Control
αCD4
CD25
CD4
FoxP3
CD4
3
1
89
90
6
5
91
88
C
(% of CD4+CD25- cells)
Control αCD4 Control αCD4
IFN γ
IL4
D
CD8 lymphocyte proliferation (% of CD8 cells)
no Tregs Control αCD4
Treg

Figure 7